Pocket Mutations of HLA-B27 Show That Anchor Residues Act Cumulatively To Stabilize Peptide Binding

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ABSTRACT: Major histocompatibility complex (MHC) class I molecules bind endogenously synthesized peptides for presentation to cytotoxic T-cells. The human class I molecule HLA-B27 consists of a trimolecular complex containing the HLA-B27 heavy chain, a peptide that is usually nine amino acid residues (aa) long, and β_2-microglobulin (β_2m). The key interactions for peptide selectivity are between Glu-45, which forms a salt bridge with the Arg at P2 of the peptide, and Asp-116 which favors the binding of peptides containing a Lys or Arg at P9. The $t_{1/2}$ of dissociation of [^{125}I]β_2m was measured for peptide-specific HLA-B27 wild-type (wt) and mutant complexes. HLA-B27 wt and HLA-B27 D116F formed relatively stable complexes, with a $t_{1/2}$ of dissociation on the scale of hours, with appropriate peptides that contained Arg at P2, whereas HLA-B27 E45T required a Gln at P2. Similarly, kinetically stable D116F complexes were formed only with peptides that contained a Leu or Val at P9 instead of Arg or Lys. The [^{125}I]β_2m dissociation rate data were fit to a set of equations in order to calculate relative binding coefficients for each anchor residue at P2 and P9. The P2 coefficients were sensitive to the E45T mutation but not the D116F mutation, whereas the P9 coefficients were sensitive only to the D116F mutation. Thus, drastic structural changes in one subsite do not affect the other subsite, indicating that the dominant anchor residues at P2 and P9 independently contribute to stabilizing the class I/peptide complex.

HLA class I molecules bind and present peptides to the T-cell receptor of CD8$^+$, CTL[1] (Townsend et al., 1989a). Endogenously processed peptides bind class I molecules in an intracellular exocytic compartment and are required for the efficient folding and assembly of class I molecules with β_2-microglobulin (β_2m) (Townsend et al., 1989b). Crystallographic analyses of three human class I molecules have indicated that the specificity of peptide binding is based on the detailed structure of pockets that extend from a central groove formed by the first 180 amino acids (aa) of the heavy chain (Bjorkman & Parham, 1990; Garrett et al., 1989; Madden et al., 1991). In all class I molecules examined so far, the peptide appears to bind in an extended conformation with the peptide amino terminus at one side of the groove in pocket A and the carboxyl terminus at the other side of the groove in pocket F (Madden et al., 1991). In the case of HLA-B27, the Arg at P2 of the peptide is shared by all peptides that copurify with HLA-B27 and is therefore thought to be a dominant anchor residue (Jardetzky et al., 1991). This arginine extends into the B pocket, forms a salt bridge with Glu-45, and also forms hydrogen bonds to Thr-24 and to a well-ordered water molecule (Madden et al., 1991; Guo et al., 1993). It has been shown for both HLA-A2 (Rötzschke et al., 1992; Matsui et al., 1993; Colbert et al., 1993) and HLA-B27 (Buxton et al., 1992; Colbert et al., 1993; Carreno et al., 1993) that mutations in the neighborhood of the B pocket affect which aa can serve as the anchor residue at P2. The second anchor residue, located at P9, is usually Lys or Arg, but it can also be Leu or Tyr in peptides that bind to HLA-B27. Asp-116, which is located at the base of the F pocket, has been proposed to form a salt bridge with basic side chains at P9 when they are present (Madden et al., 1992). A similar phenomenon was observed in the mutant HLA-A2 molecule, HLA-A2 Y116D. The Asp introduced enabled HLA-A2 to bind peptides with an Arg at P9 (Morrison et al., 1992). In the case of HLA-B27, the mutation D116F reduced the ability of HLA-B27 to bind peptides with a positively charged aa at P9 (Carreno et al., 1993). In addition, HLA-A3 and HLA-A1, which, like HLA-B27, contain Asp-116, also preferentially bind (HLA-A3) or are able to bind (HLA-A1) peptides that contain Lys at P9 (DiBrino et al., 1993a,b).

Although these data clarify the relationship between anchor residues in peptides and certain key residues in the class I peptide-binding groove, a question left unanswered is whether the broad tolerance of HLA-B27 for peptides with different aa at P9 means that the P9 anchor residue is relatively unimportant in peptide binding. It has been reported that peptides that differ only at the C-terminal anchor residue have approximately the same free energy of binding to H-2K^b, yet dissociate at different rates (Matsumura et al., 1992; Saito et al., 1993). Consequently, these authors believe that peptides containing stabilizing C-terminal anchor residues form complexes more slowly than destabilizing peptides, so as to compensate for a slower rate of dissociation (Saito et al., 1993). We considered the possibility that, in analogous fashion, the Arg at P2 might be crucial for the specificity of peptide binding to HLA-B27, whereas the P9 anchor residue might have a distinct, kinetic role in stabilizing the complex. To examine this issue, we measured the ability of 22 different peptides to bind to HLA-B27 molecules using an assay that measures the peptide-dependent formation of heterotrimeric HLA complexes that consist of iodinated β_2m, an HLA-B27 heavy-

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[1] Abbreviations: aa, amino acid residue; CTL, cytotoxic T-cell; GF, gel filtration; IBS, independent binding of side chains; MHC, major histocompatibility complex; NP, nucleoprotein; P, position; β_2m, β_2-microglobulin; $t_{1/2}$, half-life; wt, wild type.

Table 1: $t_{1/2}$ Values of β_2m Dissociation from HLA Complexes Containing Specific Peptides

		HLA-B27				HLA-B27 D116F				HLA-27B E45T				HLA-B27 T24S			
no. (A)	peptide sequence (B)	%[a] (C)	exp[b] (D)	theor[c] (E)	f[d] (F)	% (G)	exp (H)	theor (I)	f (J)	% (K)	exp (L)	theor (M)	f (N)	% (O)	exp (P)	theor (Q)	f (R)
1	SRYWAIRTK	80	1300	660	2.0	50	6	8	1.3	40	3	2	1.5	80	1600	660	2.4
2	SRYWAIRTL	50	190	220	1.2	60	120	220	1.8	7	–[e]	0.8		90	150	220	1.5
3	SRYWAIRTR	20	550	300	1.8	5	–	0.2		2	–	1		60	160	300	1.9
4	SQYWAIRTK	20	3	11	3.7	20	220[f]	0.1	2200	70	30	28	1.1	30	17	11	1.5
5	SMYWAIRTR	1	–	2		1	–	0.002		40	2	3	1.5	10	5	3	1.7
6	GRAFVTIGK	50	2700	2200	1.2	20	2	27	14	20	9	9	1.0	80	4000	2200	1.8
7	GRAFVTIGL	20	1000	720	1.4	30	1600	720	2.2	5	–	3		70	990	720	1.4
8	GQAFVTIGK	3	5	37	6.2	2	–	0.4		30	100	93	1.1	50	57	37	1.5
9	LRFGYPVYV	90	2100	1600	1.3	90	1200	1600	1.3	40	8	6	1.3	80	2100	1600	1.3
10	LQFGYPVYK	40	510	350	1.5	20	5	4	1.2	90	1400	870	1.6	90	100	350	3.5
11	LQFGYPVYV	20	20	27	1.4	50	15	27	1.8	60	100	69	1.4	80	87	27	3.2
12	LLFGYPVYK	8	–	27		8	3	0.3	10	40	58	27	2.1	80	62	27	2.3
13	LLFGYPVYV	2	–	2		20	2	2	1.0	30	2	2	1.0	20	3	2	1.5
14	LMFGYPVYV	4	–	17		20	4	17	4.3	50	71	17	4.2	50	12	17	1.4
15	GQLGFVFTK	80	290	570	2.0	50	60[f]	7	8.6	90	1500	1400	1.1	90	280	570	2.0
16	GQLGFVFTL	70	480	190	2.5	90	210	190	1.1	60	380	470	1.2	90	490	190	2.6
17	GRFGGGGGL	50	460	1100	2.4	80	1600	1100	1.5	10	–	4		60	250	1100	4.4
18	GRFGGGGGR	10	3200	1500	2.1	9	–	0.8		1	–	6		40	810	1500	1.9
19	GQFGGGGGK	10	35	56	1.6	3	6	0.7	8.5	40	92	140	1.5	60	81	56	1.4
20	GRFGGVGGV	45	410	340	1.2	80	270	340	1.3	1	–	1		70	280	340	1.2
21	GQFGGVGGV	9	2	6	3.0	40	4	6	1.5	30	10	14	1.4	70	5	6	1.2
22	GMFGGVGGV	2	–	4		20	2	4	2.0	30	2	4	2.0	30	13	4	3.3

[a] The average percentage of incorporation of [^{125}I]β_2m for these complexes using gel filtration. These values are averages obtained over many months, including several separate experiments. Not all of the peptides were tested in each experiment. [b] The experimental $t_{1/2}$ of dissociation of [^{125}I]β_2m from these complexes at 37 °C in 25 mM morpholinoethanesulfonic acid (pH 6.5)/150 mM NaCl. [c] The theoretical $t_{1/2}$ of dissociation calculated using the coefficients in Table 2. [d] The factor by which the theoretical $t_{1/2}$ differs from the experimental $t_{1/2}$. [e] – indicates that rate data could not be obtained due to limiting amounts of complexes. [f] These data were not included in the calculations whose results are shown here (see text).

chain fragment, and a peptide (Parker et al., 1992b). Mutant HLA-B27 heavy-chain fragments were constructed with single substitutions at aa 24 or 45 in pocket B or aa 116 in pocket F. In each case, the original aa in HLA-B27 was replaced by the corresponding aa from another class I molecule (HLA-B37). The rate of dissociation of complexes containing 22 different peptides and the four HLA-B27 molecules was measured. For each peptide, the $t_{1/2}$ of β_2m dissociation was set equal to the product of the parameters that quantify the contribution of the anchor residues at P2 and P9 to stabilizing the complex. Using this methodology, we found no evidence that there was any difference in function between the anchor residues at P2 and P9. Moreover, the anchor residue at P9 makes approximately the same contribution to complex stabilization whether or not the anchor residue preferences at P2 are changed by mutation of the HLA heavy chain, and vice versa.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were synthesized using an automated peptide synthesizer, as described (Parker et al., 1992c). All peptides were purified to homogeneity by C-18 reversed-phase HPLC, and their purity and molecular mass were confirmed by mass spectroscopy. All peptides utilized in these experiments are listed in Table 1.

HLA Reconstitution Assay. Specific binding of peptides to HLA-B27 molecules was assessed by reconstitution of complexes comprising iodinated β_2m, peptide, and a fragment of the HLA-B27 heavy chain (aa 1–274, comprising the entire α-1 to α-3 domains) expressed in *Escherichia coli* as previously described (Parker et al., 1992b,c). Reconstitution was assayed by gel filtration. HLA-B27 heavy-chain genes with Thr-24 changed to Ser (T24S), Glu-45 changed to Thr (E45T), and Asp-116 changed to Phe (D116F) were created by site-directed mutagenesis, as described previously (Carreno et al., 1993). HLA-B27 heavy-chain gene fragments 1–274 were generated by PCR amplification of each of the mutant heavy-chain cDNAs and were cloned into the *Escherichia coli* expression vector pHN1 (MacFerrin et al., 1990) as described (Parker et al., 1992b). All mutant genes were completely sequenced to ensure that only the intended change had been introduced. The $t_{1/2}$ of β_2m dissociation was measured by following the dissociation of [^{125}I]β_2m as a function of time using HPLC gel filtration, as described (Parker et al., 1992c). The β_2m dissociation rate varies over at least 4 orders of magnitude, depending on which peptide is bound (Parker et al., 1992c).

Presentation of NP Peptides by HLA-B27 to NP Peptide-Specific CTL. The generation and specificity of NP 383–391 (SRYWAIRTR) peptide-specific, HLA-B27-restricted CTL lines were previously described (Carreno et al., 1992). HMy.C1R cells (Storkus et al., 1987) that were transfected with a genomic clone of HLA-B27 (pE1.1, kindly provided by Dr. J. Taurog, Southwestern Medical School, Dallas, TX) or cDNA clones of HLA-B27 containing site-directed mutants of HLA-B27 were used as targets, also as previously described (Winter et al., 1991). The levels of cell surface expression of the native and mutant HLA-B27 molecules were assayed by indirect immunofluorescence with MAb ME1 (Ellis et al., 1982). HLA-B27 wt, HLA-B27 T24S, and HLA-B27 D116F had similar levels of expression, whereas HLA-B27 E45T was expressed at a lower level. CTL assays were performed by the incubation of ^{51}Cr-labeled target cells with various concentrations of synthetic peptides for 1 h at 37 °C, followed by washing and incubation for 4 h at 37 °C with effector cells in a standard ^{51}Cr-release assay (Shimojo et al., 1990). Results are reported as the percent specific lysis of triplicate determinations.

Mathematical Modeling. The theoretical $t_{1/2}$ of dissociation in minutes at 37 °C of the 22 peptide complexes (see Table 1, columns D, H, L, and F) for HLA-B27 and each of the three mutants was assumed to be proportional to the product

Table 2: Coefficients Calculated Using Equations

parameter	88 equations[a]	86 equations[b]
R2[c]	100.0[d]	100.0
L2	0.19	0.13
M2	1.2	1.0
Q2	2.5	1.7
R2 E45T	0.45	0.39
K9	100.0	100.0
L9	36	33
R9	43	46
V9	13	7.9
K9 D116F	5.9	1.2
R9 D116F	0.28	0.024
G_AFVTIG_[e]	1.0	1.0
L_FGYPVY_	6.5	9.3
G_LGFVFT_	16	15
S_YWAIRT_	0.49	0.30
G_FGGGGG_	1.3	2.0
G_FGGVGG_	1.4	1.5
nor[f]	0.16	0.22

[a] Indicates that the coefficients were obtained using all of the data in Table 1. [b] Indicates that the data for HLA-B27 D116F complexes containing SQYWAIRTK and GQLGFVFTK were ignored in order to improve the fit of the remaining data. [c] R2 refers to the coefficient for arginine at P2 of the peptide. [d] The numbers in each column were normalized to R2 = 100, K9 = 100, or G_AFVTIG_ = 1. The experimental $t_{1/2}$'s listed in Table 1 were obtained by multiplying the appropriate coefficients in the 86 equations column. Thus, the theoretical $t_{1/2}$ for HLA-B27, HLA-B27 T24S, and GRAFVTIGK is 100 × 100 × 1 × 0.22 = 2200 min. [e] _ in the peptide sequence indicates that one of the aa listed in the parameter column is to be substituted at this position. [f] The overall normalization constant.

of three parameters: a parameter that represents the relative binding ability of the aa at P2 and corresponding parameters for the relative binding abilities of the aa at P9 and of the remainder of the peptide (that is, the aa at P1 and P3–P8). An overall normalization parameter (*nor*, see below) served to convert to units of minutes at 37 °C. Numerical coefficients for these parameters were calculated by solving 88 simultaneous equations (Parker et al., 1994), corresponding to each of the four HLA molecules with 22 different peptides. If no complex could be formed, or if the complex that formed had a $t_{1/2}$ of dissociation of <5 min, the data were considered to fit perfectly if the theoretical $t_{1/2}$ was also <5 min.

The following assumptions were made prior to the calculations, regarding the parameters to be determined (listed in Table 2). The soundness of these assumptions is supported by the fit of experimental $t_{1/2}$ to theoretical $t_{1/2}$ (see Table 1). A single parameter for Gln at P2 was assumed to apply to all four HLA-B27 molecules. Likewise, Leu and Met at P2 were assigned one parameter each. In contrast, Arg at P2 was split into two parameters: one parameter that applied to HLA-B27, HLA-B27 T24S, and HLA-B27D116F, and a second parameter that applied to HLA-B27 E45T only. Similarly, at P9, the coefficients for Leu and Val were assigned parameters that applied to all four HLA-B27 molecules, but two different parameters were assigned to each of Lys and Arg, one of which applied to HLA-B27 D116F only. In addition, six different parameters were assigned each to the possible peptide sequences at P1 and P3–P8 (subsequently referred to as the peptide context). Finally, there is the overall normalization parameter, which converts the theoretical $t_{1/2}$ of dissociation to units of minutes at 37 °C.

In this scheme, only 15 of the described parameters are really independent. There are a sufficient number of degrees of freedom in these calculations such that one parameter at P2, one at P9, and one peptide context parameter can be assigned arbitrarily. We chose to set the coefficient for Arg at P2 to 100.0, that for Lys at P9 to 100.0, and the peptide context G_AFVTIG_to 1.0. Thus, the peptide with sequence GRAFVTIGK, which is known to form stable HLA-B27 complexes (Parker et al., 1992c), contains all three arbitrarily defined parameters, and the overall normalization constant (*nor*) becomes equal to one ten-thousandth [1/(1 × 100 × 100)] of the theoretical $t_{1/2}$ of β_2m dissociation from HLA-B27/GRAFVTIGK complexes. In addition, the values of the other coefficients become normalized to Arg at P2, Lys at P9, or the context G_AFVTIG_.

The coefficients were calculated using a Fortran program (Parker et al., 1994) that searched for the best values of the coefficients so as to minimize the overall error, which was set equal to the sum of the errors for each data point: error = $[\log(\text{experimental } t_{1/2}) - \log(\text{theoretical } t_{1/2})]^2$. For a peptide of sequence SRYWAIRTR, theoretical $t_{1/2}$ = R2 × R9 × S_YWAIRT_ × *nor*, where R2 is the coefficient for Arg at P2, R9 is the coefficient for Arg at P9, S_YWAIRT_ is the coefficient for peptides containing Ser at P1 and Tyr, Trp, Ala, Ile, Arg, and Thr at P3–P8, respectively, and *nor* is the overall normalization constant that converts $t_{1/2}$ into units of minutes at 37 °C.

RESULTS

Binding of Peptides to HLA-B27. We have previously demonstrated that the antigenic HLA-B27-restricted influenza nucleoprotein peptide SRYWAIRTR (Huet et al., 1990) and the HIV gp120 peptide GRAFVTIGK bind to HLA-B27 *in vitro*, as assessed by the incorporation of radiolabeled β_2m into HLA-B27 complexes (Parker et al., 1992b). These data are listed in Table 1 as peptides 3 and 6 in column C; subsequent references to Table 1 will list the row number and column number, in this case [3C] and [6C]. The HLA-A2-restricted HTLV-1 tax peptide LLFGYPVYV (Utz et al., 1992) does not bind to HLA-B27 [13C]. However, when the Leu at P2 is replaced by Arg to form LRFGYPVYV, complexes are formed that dissociate with a $t_{1/2}$ of 2100 min at 37 °C [9D], which are nearly as stable[2] as complexes formed with GRAFVTIGK ($t_{1/2}$ = 2700 min [6D]) and about 4-fold more stable than SRYWAIRTR complexes ($t_{1/2}$ = 550 min, [3D]). Likewise, the HLA-A2 binding poly(Gly)-based peptide GLFGGVGGV (Parker et al., 1992a) can be converted into an HLA-B27 binding peptide by substituting the Leu at P2 with Arg to form GRFGGVGGV ($t_{1/2}$ = 410 min [20D]).

Binding of Peptides to HLA-B27 D116F. HLA-B27 D116F was prepared in order to assess the importance of the expected salt bridge (Madden et al., 1992) between Asp-116 and the basic residues that are present at P9 in some HLA-B27 binding peptides (Jardetzky et al., 1991). It was found that neither SRYWAIRTR [3G] nor GRAFVTIGK [6G] could form stable complexes with HLA-B27 D116F. However, when the positively charged C-terminal residue of these peptides was converted to Leu, the peptides were able to bind to HLA-B27 D116F ($t_{1/2}$ = 120 min [2H] and $t_{1/2}$ = 1600 min [7H]), as well as to HLA-B27 wt ($t_{1/2}$ = 780 [2D] and 1000 min [7D], respectively) [see also Carreno et al. (1993)]. As expected, LRFGYPVYV and GRFGGVGGV could also bind to HLA-B27 D116F ($t_{1/2}$ = 1200 min [9H] and $t_{1/2}$ = 270 min [20H]). In order to demonstrate that these findings

[2] Throughout this paper, whenever we refer to the stability of HLA/peptide/β_2m complexes, we mean the kinetic stability of the complex to dissociation, not the free energy of peptide binding or the peptide binding affinity.

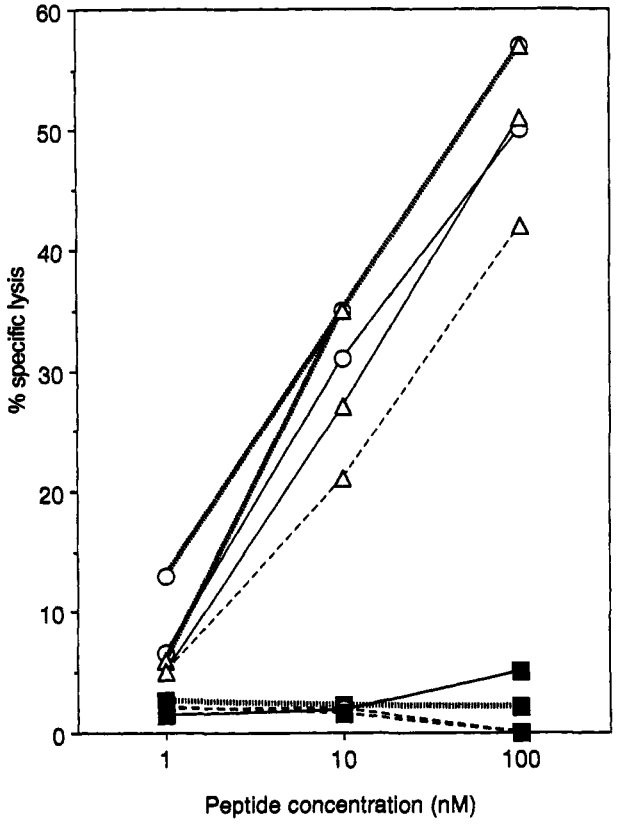

FIGURE 1: Presentation of influenza A NP peptides to CTL by HLA-B27, HLA-B27 D116F, and HLA-B27 T24S bearing cells. HMy.C1R cells transfected with wild-type HLA-B27 (—), HLA-B27 T24S (···), or HLA-B27 D116F (- - -) were incubated with the indicated concentrations of SRYWAIRTR (○) , SRYWAIRTL (△), or SKYWAIRTR (■) for 1 h at 37 °C. The cells were then washed, incubated at 37 °C for 4 h, washed once, and assayed for susceptibility to lysis by the HLA-B27-restricted NP peptide-specific (SRYWAIRTR) CTL line 1B12 at an E:T ratio of 0.5:1.

have biological relevance, CTLs specific for the HLA-B27/SRYWAIRTR complex were tested for their ability to recognize SRYWAIRTL. As shown in Figure 1, the CTL could recognize SRYWAIRTL on HLA-B27 D116F target cells but not SRYWAIRTR, in agreement with the binding data. Moreover, the substitution of Arg with Leu at P9 did not affect recognition by CTL on HLA-B27 wt cells. As an additional peptide specificity control, note that SKYWAIRTR could not sensitize HLA-B27 target cells, either because it could not bind or because the epitope was changed. The fact that SKYWAIRTR does not detectably form HLA-B27 complexes (data not shown) indicates that the former explanation is likely to be correct.

Binding of Peptides to HLA-B27 E45T. HLA-B27 E45T was prepared in order to investigate the role of the salt bridge between Glu-45 and the Arg at P2 in stabilizing HLA-B27 complexes. It was found that SRYWAIRTR could not detectably bind to HLA-B27 E45T [3K], whereas GRAFVTIGK formed small amounts of complexes [6K] that dissociated rapidly ($t_{1/2}$ = 9 [6L] vs 2700 min for HLA-B27 [6D]).

We had previously synthesized a series of peptides substituted at P2 (including all possible aa except Trp and Cys) within the context GXFGGVGGV to determine which aa could substitute for the dominant anchor residue at P2 that is required for peptide binding to HLA-A2 (Parker et al., 1992a). These same peptides were tested with HLA-B27 E45T to determine whether any aa at P2 could compensate for the E45T mutation. It was found that both GQFGGVGGV [21K] and GMFGGVGGV [22K] could form detectable complexes (30% β_2m incorporation), but GRFGGVGGV [20K] and GLFGGVGGV (data not shown) were unable to bind above background. Rate constant determinations revealed that the complexes containing GQFGGVGGV ($t_{1/2}$ = 10 min [21L]) were significantly more stable than those containing GMFGGVGGV ($t_{1/2}$ = 2 min [22L]). No other aa at P2 promoted detectable complex formation in this peptide context, either for HLA-E45T or for unmutated HLA-B27 (data not shown).

Because Gln served as an effective P2 anchor residue in the peptide GQFGGVGGV, we sought to determine whether other peptides that bound to HLA-B27 but not HLA-B27 E45T could be converted to HLA-B27 E45T binding peptides if Gln were substituted for the Arg at P2. In addition, a Gln-substituted analog of the HLA-A2-restricted influenza matrix peptide GILGFVFTL (Morrison et al., 1992) was prepared. In each case, the Gln peptides did indeed form stable HLA-B27 E45T complexes (GQAFVTIGK, $t_{1/2}$ = 100 min [8L]; LQFGYPVYV, $t_{1/2}$ = 100 min [11L]; GQLGFVFTL, $t_{1/2}$ = 380 min [16L]). Two Met analog peptides were also prepared. Both LMFGYPVYV and SMYWAIRTR formed HLA-B27 E45T complexes that had relatively slow dissociation rates ($t_{1/2}$ = 71 [14L] and 25 min [5L], respectively).[3]

Binding of Peptides That Contain Gln at P2 to HLA-B27. Having established that peptides that contain Gln at P2 can bind to HLA-B27 E45T, we also tested these peptides with wild-type HLA-B27. We found, to our surprise, that all of these peptides bound to HLA-B27 [column D, 4, 8, 10, 11, 15, 16, 19, 21] and that furthermore, in the case of GQLGFVFTL [16D], the complex formed was comparable in stability to the corresponding HLA-B27 E45T complex [16L]. In addition, the peptides that contained Gln at P2 also bound to HLA-B27 D116F, provided that an acceptable anchor residue was present at P9 (GQLGFVFTL, $t_{1/2}$ = 210 min [16H]; LQFGYPVYV, $t_{1/2}$ = 20 min [21H]).

Binding of Peptides to HLA-B27 T24S. Both of the previous mutants described above dramatically alter the peptide binding motif for HLA-B27. To determine whether a conservative mutation in the B pocket would have any effect on peptide binding, we examined mutant HLA-B27 T24S. Thr-24 is known to make a hydrogen bond to the Arg at P2 of the peptide (Madden et al., 1992), and when it is replaced by Ser, the same hydrogen bond presumably could be made, although the pocket would be larger by the size of a methyl group. We found that this substitution had very little impact on both peptide selectivity and the stability of complexes once formed. Of the 17 peptides for which kinetic data could be obtained, 9 form complexes whose $t_{1/2}$ values for HLA-B27 and HLA-B27 T24S [D and P] are within a factor of 2 [1, 2, 6, 7, 9, 15–17, 20]. In two other cases, the $t_{1/2}$'s were too fast to measure accurately and/or very little complex could be formed [4, 21], so that no informative comparison could be made. The most discordant result was a 10-fold difference in dissociation rate for GQAFVTIGK [8D,P]. Surprisingly, we have also observed that HLA-B27 T24S complexes can often be made in higher yield than wild-type HLA-B27 complexes [O vs C, 2, 3, 5-8, 10–14, 18–22]. It is possible that HLA-B27 T24S binds peptide and β_2m more rapidly

[3] Two peptides containing Lys at P9 (SQYWAIRTK [4H] and GQLGFVFTK [15H]) behaved erratically when complexes were prepared. Out of five experiments with SQYWAIRTK, in two cases the complexes dissociated rapidly (<5 min, data not shown) and in three other experiments a portion of the complexes was rather stable (53–330 min). Similar data were obtained for GQLGFVFTK. So far, we have not been able to resolve the basis for these inconsistencies.

under our reconstitution conditions, possibly because the HLA-B27 T24S preparation contains more correctly oxidized heavy chains. Interestingly, target cells expressing HLA-B27 T24S and treated with SRYWAIRTR were recognized slightly better by SRYWAIRTR-specific T-cells than were wt cells (Figure 1), indicating that at the cell surface as well HLA-B27 T24S may have some advantage in efficiency over wt HLA-B27. This may also explain why some peptides that contained Met or Leu at P2 bound significantly to HLA-B27 T24S [12–14,22O], but not to HLA-B27 wt [12–14,22C].

Fitting of the Data to a Mathematical Model Based on Independent Binding of aa. The above data indicate that, in general, peptides that contain Arg at P2 bind tightly to HLA-B27, HLA-B27 T24S, and HLA-B27 D116F, but not to HLA-B27 E45T. Similarly, peptides that contain Lys or Arg at P9 bind well to HLA-B27, HLA-B27 T24S, and HLA-B27 E45T, but not to HLA-B27 D116F. In contrast, peptides that contain a Gln at P2 or a Leu at P9 bind to all four HLA-B27 molecules. We created a mathematical model to determine whether there is a single ranking order for anchor residue preferences at P2 and P9 that would be maintained given any peptide context (i.e., the aa at P1 and P3–P8). In addition, the model addresses how changes in key pocket residues affect the ranking order of anchor residues. The model was designed to quantitate these binding preferences and was based on the assumption that the $t_{1/2}$ of dissociation for each complex is determined by independent contributions from each aa at each position within the peptide. This idea previously has been successfully applied to a study of the binding capacity of 160 different peptides for HLA-A2 (Parker et al., 1994). In the present study, we assigned individual coefficients only to the dominant anchor residues at P2 and P9, because the nonanchor residues were held constant within sets of peptides. In every case, the theoretical $t_{1/2}$ of dissociation was assumed to be dependent on three different factors: the aa at P2, the aa at P9, and the peptide context (which was a function of the remaining aa). It was therefore necessary to calculate 18 coefficients (see Table 2): one for each aa at P2 and at P9, and for each peptide context. We found that in most cases the calculated theoretical $t_{1/2}$ was quite close to the experimentally measured $t_{1/2}$ [compare Table 1 columns D and E, H and I, L and M, and P and Q]. For only 10 out of 88 peptides was the difference in $t_{1/2}$ greater than a factor of 4 [see columns F, J, N, R].

The relative ability of each of the anchor residues to support peptide binding to each of the HLA-B27 molecules is evident from the values of the coefficients listed in Table 2. The data indicate that Gln at P2 is about 40-fold worse than Arg for binding to HLA-B27, HLA-B27 T24S, and HLA-B27 D116F, whereas Met is 80-fold worse and Leu is approximately 500-fold worse. Interestingly, when Glu-45 is substituted with Thr, the ability of Leu, Met, and Gln at P2 to contribute to peptide binding is virtually unchanged, whereas Arg at P2 becomes 200-fold less acceptable. A similar phenomenon is found at P9. Leu and Arg are only 2–3-fold less optimal than Lys at P9 in HLA-B27, HLA-B27 T24S, and HLA-B27 E45T, whereas Val at P9 is about 8-fold worse. The ability of Leu and Val at P9 to contribute to peptide binding is unaltered when Asp-116 is substituted with Tyr, but Lys and Arg become more than 40-fold less acceptable. An examination of the calculated values for the peptide context parameters reveals that G_LGFVFT_ and L_FGYPVY_ are 5–20-fold better than G_AFVTIG_, while S_YWAIRT_ is about 2-fold worse. The two poly(Gly)-based contexts (G_FGGGGG_ and G_FGGVGG_) are nearly equivalent to one another and to G_AFVTIG_.

As the data were being collected, as mentioned in footnote 3, there was difficulty in obtaining a credible $t_{1/2}$ of dissociation for two peptides (SQYWAIRTK and GQLGFVFTK) to HLA-B27 D116F. To determine how sensitive the calculations would be to this kind of experimental problem, we compared the coefficients calculated using all 88 data points (Table 2, column 2) with the coefficients calculated using the 86 internally consistent data points (Table 2, column 3). The coefficients had similar values in both cases, indicating that the calculations are "robust" and are not easily disrupted by discordant data. The overall difference between experimental and theoretical data was about twice as great when all 88 peptides were used (data not shown), so that each of the remaining 86 peptides contributed about one-forty-third as much to this difference as did these two peptides. Because it cannot yet be ruled out that the HLA-B27 D116F data for SQYWAIRTK and GQLGFVFTK might be caused by an unknown structural modification that occurred during storage or handling of the peptide, which affects peptide binding to HLA-B27 D116F, all of the theoretical rate data in Table 1 were calculated disregarding the rate data for these two data points.

DISCUSSION

All peptides that form stable complexes with a given MHC class I molecule share certain aa, defined as anchor residues, that bind in pockets of the peptide binding groove (Falk et al., 1991; Garrett et al., 1989; Madden et al., 1993). For any given peptide, at any anchor position, the 20 natural aa can be ranked in a hierarchy. The highest ranking aa in the hierarchy will be the anchor residue that is best able to stabilize the class I molecule/peptide complex, assuming the other aa in the peptide are left unchanged. At an important anchor position, a low-ranking aa would be expected to prevent peptide binding altogether. An important issue is whether the hierarchy of acceptable anchor residues at a given position is the same for all peptides to the first approximation. A second, related issue is the mechanism by which multiple anchor residues contribute to stabilizing the class I molecule/peptide complex. In the simplest case, the anchor residues would cumulatively contribute to peptide binding. We have called this independent binding of side chains (IBS) (Parker et al., 1994). If the side chains bound roughly independently, a peptide that contained a tolerable but low-ranking anchor residue could be converted to a more tightly binding peptide by substitutions according to the hierarchies of acceptable aa at other anchor positions. At the other extreme, anchor residues might not act independently; instead, they might interact with one another in a complicated way. For example, the binding of a dominant anchor residue might initiate conformational changes that must take place prior to the binding of auxiliary anchor residues. A third question deals with the consequences of mutations in the pockets of the class I molecule that accommodate anchor residues. A mutation might affect the hierarchy of preferences only for those anchor residues that directly interact with it. Alternatively, through conformational changes, mutations might affect the peptide binding ability of other anchor residues that bind in distinct parts of the peptide binding groove. On the basis of the IBS assumption, we developed an algorithm that calculates the portion of the overall binding stability that is contributed by each anchor residue. Basically, the binding data for each peptide are converted into a mathematical equation in which the measured $t_{1/2}$ of dissociation of the peptide complex is set equal to the product of coefficients that represent the

contribution of each anchor residue to the stability of the complex. These coefficients are calculated by simultaneously solving the equations that correspond to the binding data for a large number of peptides.

The X-ray crystal structure of HLA-B27 shows that Arg, which has invariably been found at P2 in HLA-B27 binding peptides (Jardetzky et al., 1991), extends into pocket B of the peptide binding site (Madden et al., 1992). The P9 aa, however, can be any one of a number of aa and is oriented toward pocket F. Both CTL data and peptide binding data using whole cells have indicated that individual residues in the B and F pockets are important for peptide binding (Buxton et al., 1992; Colbert et al., 1993; Carreno et al., 1993). For example, iodinated influenza A nucleoprotein peptide 383–391 SRYWAIRTR bound to HLA-B27 wt at the cell surface but less well to HLA-B27 D116F. In contrast, two peptides with a C-terminal Leu, SRYWAIRTL and RRYQKSTEL, bound comparably to both molecules (Carreno et al., 1993). Presumably, mutation of Asp-116 eliminated a salt bridge that was necessary for binding peptides with basic residues at P9. A similar situation was described for HLA-A2. CTL from individuals previously infected with influenza A originally recognized peptide GILGFVFTL as the optimal antigenic peptide, but when Tyr-116 in HLA-A2 was mutated to Asp, GILGFVFTR was preferentially recognized (Morrison et al., 1992).

HLA-B27 is a good candidate molecule to study the relationship between anchor residue hierarchies and binding pockets because it is unusual in its exclusive requirement for Arg at P2. Our approach was to compare the dissociation rates of a large number of different H chain/β_2m/peptide complexes. Because dissociation reactions are first-order reactions, no reagent concentrations need to be determined, and consequently the $t_{1/2}$ of dissociation can be measured accurately. Equilibrium constants, on the other hand, are hard to measure accurately in this system, because a long time would be required to reach equilibrium starting from complexes with a $t_{1/2}$ of dissociation that is often in the range of days at 37 °C. The $t_{1/2}$ of class I/peptide complexes can be determined easily by following the dissociation of ^{125}I-labeled β_2m (Parker et al., 1992c). The same [^{125}I]β_2m preparation can be used to study any class I molecule/peptide interaction, and one can obtain homogeneous class I molecule/peptide/β_2m complexes by starting with denatured HLA class I heavy chains synthesized by *E. coli*, which do not bind β_2m unless peptides are intentionally added (Parker et al., 1992b).

Using this approach, we found that when the salt bridge between the usually invariant Arg at P2 of the peptide was disrupted by mutation (E45T), complexes were formed that were, according to the coefficients in Table 2, on the average 200-fold less stable than the corresponding wt HLA-B27 complexes. However, if the peptides contained Gln at P2, the complexes were 4–10-fold more stable than the corresponding Arg peptides. Thus, Gln at P2 can partially compensate for the E45T mutation. The Gln peptides were also able to bind to wt HLA-B27 and HLA-B27 molecules that had mutations in parts of the peptide binding groove other than pocket B. Presumably, the planar amide group of the Gln side chain is able to make H-bonds that function analogously to the H-bonds of the planar guanidyl group of the Arg side chain. Our failure to detect any binding with Asn-containing peptides may indicate that the Asn side chain does not reach far enough into the B pocket to make productive H-bonds.

The ability of Met, and to a lesser degree Leu, to substitute for Arg in binding to HLA-E45T and HLA-B27 T24S indicates that other binding phenomena such as van der Waals interactions may be important. Although our data indicated that Gln, and possibly even Met and Leu, could be tolerated at P2 if the remainder of the peptide is ideal for binding to HLA-B27, no endogenous peptides have yet been isolated that contain these residues at P2 (Jardetzky et al., 1991). Presumably, such peptides are present in the endogenous peptide repertoire, but are rare because Arg can satisfy the binding requirements with fewer constraints on the rest of the peptide's sequence. This probably explains the lower cell surface expression found for HLA-B27 E45T compared to the other HLA-B27 molecules that we have studied (Carreno et al., 1993). One might expect that peptides that contain Gln at P2 would be more plentiful among the endogenous peptides associated with HLA-B27 E45T molecules. Such a peptide may be the basis for the finding that one allo-CTL was able to recognize both HLA-B27 wt and HLA-B27 E45M (Buxton et al., 1992).

Arg, Lys, Leu, Ala, and Tyr were identified at P9 in endogenous peptides isolated from HLA-B27 (Jardetzky et al., 1991), suggesting less importance for the C-terminal aa, as has been proposed for H-2K^b (Saito et al., 1993). We believe that this conclusion is unwarranted for HLA-B27, from the following argument. HLA-B27 D116F discriminates against peptides that contain Lys, Arg, or Tyr (data not shown) at P9. On this account, one might expect that peptides that contain Leu at P9 would bind more tightly to HLA-B27 D116F than to HLA-B27 wt or that the importance of the P2 anchor residue would be altered in HLA-B27 D116F compared to HLA-B27 wt. However, neither of these expectations is supported by the data. First, HLA-B27 D116F and wt complexes were found to have a similar range of values for $t_{1/2}$ of dissociation. Second, a single set of coefficients for Leu at P9, Val at P9, and Arg at P2 that applied to both HLA-B27 wt and HLA-B27 D116F resulted in a good fit to the data. Therefore, the degree of stabilization of binding provided by P9 side chains to HLA-B27 wt and D116F is similar and presumably is also mechanistically similar to that provided by the P2 side chains. In fact, Gln at P2 and Leu at P9 are, to the first approximation, equally acceptable for all four molecules tested.[4] These data argue against the possibility that dramatic conformational changes in HLA-B27 take place upon peptide binding. The finding that mutations affecting residues that are most important for peptide selectivity result in an altered peptide binding motif has evolutionary implications. The present repertoire of class I molecules in the human population appears to be limited in that so far natural molecules like HLA-B27 E45T and HLA-B27 D116F have not been identified, even though they could easily be generated from other class I molecules that have the aa in question. Thus, the ability to bind peptides is not the only consideration of importance in class I molecule evolution.

Ideally, the larger the amount of data, the better one could predict the peptide binding behavior of an untested peptide. In reality, it is not straightforward to make such predictions because of the number of variables that come into play and

[4] When the coefficient for Gln at P2 was allowed to be different for HLA-B27 E45T than for the other three molecules, the fit of the theoretical $t_{1/2}$ to the experimental $t_{1/2}$ improved slightly (data not shown). The values of the other coefficients changed slightly, and the coefficient for Gln at P2 for HLA-B27 E45T became about twice as great as those for the other three molecules, indicating that peptides that contain Gln at P2 may bind slightly better to HLA-B27 E45T. However, the extent of this improvement was small and was similar in magnitude to the improvement that could be anticipated with any increase in the number of variables.

the shear volume of the data. The mathematical modeling that we have developed was designed to solve this problem. Using this approach, all of the available peptide binding data are impartially entered as input, and the contributions of individual aa are calculated on athe basis of a set of specific assumptions about what factors control peptide binding. In this article, we have assumed that the $t_{1/2}$ of dissociation was determined by three factors that act independently: the anchor residue at P2, the anchor residue at P9, and the peptide context. We believe that the correspondence between the theoretical $t_{1/2}$ and experimental $t_{1/2}$ in Table 1 verifies our model.

We anticipate that on theoretical grounds there should be second-order effects, such as significant peptide side-chain/side-chain interactions, that in some cases will result in a poor correspondence between experimental data and the model. In addition, some individual measurements may be in error. Without additional experimentation, one cannot distinguish real second-order effects from purely experimental limitations. However, a poor prediction is reason to suspect an experimental problem, such as a chemically modified peptide contaminant that can outcompete the intended peptide in forming HLA complexes. Peptide contaminants of this sort have been shown to be the active species in earlier experiments with whole cells (Schumacher et al., 1991), making it essential that one use peptides of the highest quality. Although our peptides were purified rigorously by preparative HPLC and analyzed carefully for the presence of contaminants, we still cannot be confident that peptide heterogeneity is never a problem. Some chemical modifications to the peptide may take place during storage or in the reconstitution process itself. This may explain why SQYWAIRTK and GQLGFVFTK bind so well to HLA-B27 D116F in some experiments but not in others. In conclusion, the mathematical parametrization methodology that we have developed has allowed us to elucidate the general trends and determine when second-order effects need to be invoked to explain the binding behavior of exceptional peptides.

The overall binding energy of a peptide is theoretically equal to the sum of the binding energies contributed by each peptide residue. In practice, however, it is difficult to calculate individual residue binding energies. A significant fraction of the binding energy is probably contributed by the peptide backbone and termini (Madden et al., 1992), even though the specificity of peptide binding to class I molecules is determined by dominant anchor residues (at P2 and P9 for both HLA-A2 and HLA-B27) and auxiliary anchor residues at other positions (Deres et al., 1992; Jameson et al., 1992; Parker et al., 1992a; Ruppert et al., 1993; Saito et al., 1993). Recently, Saito et al. (1993) concluded that the majority of the binding energy was in fact contributed by the anchor residues, rather than by the peptide termini. These arguments become complicated because certain aa contribute positively to peptide binding (positive binding energy), while others are detrimental to peptide binding (negative binding energy, with respect to alanine) (Boehncke et al., 1993). In the case of HLA-A2, we have quantified the contribution of each aa in the peptide to complex stability, assuming IBS as a first approximation, so that the binding behavior of every nonamer can be predicted by the use of a table of coefficients (Parker et al., 1994). The range in values of the sequence-specific coefficients in Table 1 indicates that peptide aa, in addition to those at P2 and P9, are also important for the stability of HLA-B27 complexes. The aa that make up the peptide context stabilize or destabilize the HLA-B27 complex by a factor of at least 30. Presumably, if enough data were obtained, the peptide context could be broken down into contributions by additional aa. As we found for HLA-A2 (Parker et al., 1994), it is likely that P3 and P7 are the next most important positions.

The IBS question has been discussed with respect to other class I molecules as well. Recently, Huczko et al. (1993) found that the anchor residues for HLA-B7 also contributed separately to overall peptide binding, although the authors suggested that certain combinations of auxiliary anchor residues may be more favorable than would be expected if they acted completely independently. Likewise, Saito et al. (1993) found that, for H-2K^b, Ile could serve better as an auxiliary anchor residue at P2 when Phe instead of Tyr was present as the dominant anchor residue at P5. We have seen similar phenomena with HLA-A2, where it appears that for a small minority of peptides, significant side-chain/side-chain interactions are important (Parker et al., 1994). In these cases, two separate classes of side-chain/side-chain interactions have been observed: competitive effects where two side chains compete for the same space (Saito et al., 1993; Huczko et al., 1993; Parker et al., 1994) and cooperative effects, where two side-chains interact so as to stabilize binding (Parker et al., 1994). Both kinds of interactions may also involve readjustments in the conformation of side chains within the peptide binding groove of the class I molecule, which have been detected in HLA-A2/peptide complexes whose structures have been determined by X-ray crystallography (Madden et al., 1993).

The IBS approximation provides a basis for estimating which peptides can stabilize class I complexes the most. Our data indicate that in HLA-B27/peptide complexes, there is very little interaction between the B and F pockets. Thus, for HLA-B27 at least, to the first approximation anchor residues at P2 and P9 act independently and cumulatively in stabilizing peptide binding. We have shown that this is still true, even if either of the key residues of HLA-B27 that form salt bridges to the peptide is disrupted by mutation. We propose that for any class I molecule, it should be possible to estimate the relative binding ability of each aa at anchor positions so that the best candidates for high-affinity binding peptides can be readily identified.

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